Enduring Effects of Perinatal Nicotine Exposure on Murine Sleep in Adulthood

Research Thesis

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By

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Abstract

Epidemiological evidence shows that humans prenatally exposed to nicotine are at risk for persistent sleep problems, as well as mood and anxiety disorders. Even if the mother is not inhaling nicotine directly, second-hand smoke remains a public health issue and the danger of second-hand vapor is currently unknown. Therefore, there is high potential for a fetus to receive nicotine exposure while *in utero*. To explore this relationship between perinatal nicotine exposure and offspring sleep, breeding pairs of C57BL/6J mice were administered either vehicle (veh) or nicotine (pNic) solutions through their drinking water. This administration persisted throughout pregnancy and terminated at weaning, and all testing occurred during offspring adulthood. pNic males displayed less total movements than veh males during Open Field Exploration testing, with the strongest statistical significance seen at the start of testing. However, additional testing (Forced Swim, Elevated Plus Maze, Sucrose Preference) had no statistical significance. After observing locomotor changes in males, I selected a subset for transmitter implantation and sleep analysis by EEG/EMG telemetry. Baseline recording revealed reduced wakefulness duration and increased non-REM (NREM) sleep duration in pNic, although NREM "deepness" was the same between treatment groups. After 5 hours of sleep deprivation, pNic had a stronger rebound consisting of longer and deeper NREM sleep compared to veh and compared to both pNic and veh sleep before deprivation. Following mock immune challenge by lipopolysaccharide, veh displayed normal sleep responses whereas pNic had a blunted response. The data suggests that nicotine exposure restricted to gestation and weaning has long-term consequences on sleep that persist into adulthood. Future studies should determine a mechanism and the phenotypes in females.

1. Introduction

Smoking during pregnancy remains a public health concern with consequences to the offspring. Approximately 10% of women smoke cigarettes while pregnant (5). Amongst those who do not smoke cigarettes or use nicotine-containing substances, approximately 25% of people in the United States had evidence of exposure to second-hand smoke (28). Therefore, regardless of whether a mother is smoking while pregnant, the fetus is at risk for problems related to early developmental nicotine exposure.

Compared to cocaine, opiates, cannabis, and alcohol use, prenatal nicotine exposure had the strongest correlation with childhood sleep problems (68). Sleep abnormalities are often co-morbid with other psychiatric conditions (4), so it is possible that those prenatally exposed to nicotine may be at risk for psychiatric disorders. Indeed, prenatal nicotine exposure is correlated with affective and anxiety disorders (42, 71) as well as attention-deficit/hyperactivity disorder (ADHD) (72) in humans. However, smoking during pregnancy is also highest amongst mothers with psychiatric disorders (23), lower socioeconomic status, and maladaptive parenting styles (15). All of the aforementioned confounds are also correlated with offspring sleep problems (24, 67, 54), and experimentation is therefore necessary to characterize the effects of perinatal nicotine exposure on sleep.

There is evidence that nicotine has the ability to alter behaviors related to biological rhythms. For example, nicotine phase-shifts neuronal activity in slices of rat suprachiasmatic nucleus (SCN) (73), the region of the hypothalamus that generates and regulates the body's biological rhythms (38). Nicotine has high affinity for a subset of cholinergic receptors known as

nicotinic acetylcholine receptors (nAChR) (32), and activation of both muscarinic and nicotinic receptor subtypes through carbachol administration phase-shifts adult rats (80). Moreover, prenatal nicotine administration delays arousal in both sleeping lambs and sleeping human infants, with implications for Sudden Infant Death Syndrome (26, 19). One previous study characterized the altered sleep-wake states in perinatally-exposed offspring rats during their neonatal period of development (20). However, little is known about the long-lasting effects of perinatal nicotine exposure on sleep and behavior pathology. Sleep is an important factor to both physical and mental health. To name just a few associated pathologies, poor sleep and circadian disruptions are linked to cardiovascular disease (48), obesity (17), and neuropsychopathologies (31). Therefore, it is important to understand any potential long-term consequences of gestational/early-life exposure to nicotine. To investigate, I bred C57BL/6J mice and administered either nicotine solution or vehicle through the drinking water, and immediately terminated administration after weaning. Drug administration lasted approximately 6 weeks, persisting through the periods of conception, pregnancy, and weaning. Offspring mice aged under normal conditions for at least 9 weeks, and were then tested for measures of depressive- and anxiety-like behavior, after which a subset of mice underwent sleep recording and analysis. I hypothesized that developmental exposure to nicotine would increase anxiety and depressive-like behaviors, and would also alter baseline sleep patterns, homeostatic response to sleep deprivation, and sleep patterns following an immune challenge.

2. Methods

2.1 Animals and Drug Administration

Upon arriving at our laboratory, adult (> 8 weeks old) male and female C57Bl/6J mice were group housed and allowed to acclimate to a 14:10 light/dark cycle for 1 week prior to pairing. Mice were paired and allowed to mate for a 10-day period after which the male was removed from the cage. On the first day of pairing, mice received a water bottle containing 200 μg/ml nicotine bitartrate dihydrate (Nic; calculated as free base; MP Biomedicals, Solon, OH) in 2% w/v saccharin or 2% saccharin with 0.2% (v/v) tartaric acid (Veh; Sigma-Aldrich, St. Louis, MO), with all solutions pH-matched at 7.4 (as described by 34). Solutions were refrigerated at 4 degrees celsius until use, and breeding pairs received fresh solution every two weeks. This form of administration was chosen to avoid any gestational stress associated with osmotic mini-pump implantation (59), and to also generalize results to all forms of nicotine, rather than just smokeable tobacco. Mice had *ad libitum* access to one of these solutions, food (Harlan Teklad #7912), a cotton nestlet, and a plastic piece of housing enrichment throughout pregnancy and prior to weaning. Dams were weighed weekly to determine the approximate date of conception and ensure healthy development (data not shown). At weaning, offspring were sexed, separated, and solutions were immediately replaced with normal filtered drinking water. Animals aged normally into adulthood (>9 weeks) before any further testing took place. The experimental design described above is detailed in **Figure 1, A**. Groups numbers were as follows: males, n = 15 veh, 23 pNic; females, n = 13 veh, 16 pNic for behavioral testing; and n = 8 veh, 7 pNic males for sleep measures. All procedures were approved by The Ohio State Institutional Animal Care and Use Committee (IACUC).

2.2 Cotinine Assay

Cotinine is the primary active (i.e., capable of binding nicotinic acetylcholine receptors) metabolite of nicotine, and has a half-life of approximately 55 minutes in mice (81). Therefore, assaying concentrations of cotinine in a biological sample provides a more temporally stable assay of nicotine exposure than nicotine, which has a half-life of 6-7 min in mice (43). I measured maternal serum cotinine concentrations at weaning to confirm offspring nicotine exposure using a commercially available enzyme immunoassay kit (Calbiotech mouse/rat cotinine ELISA). Serum samples were run neat, read on a plate reader (Molecular Devices, SpectraMax Plus), and cotinine concentrations were quantified via comparing unknown optical densities against a standard curve (4-parameter logistic). Although I did not measure cotinine concentrations in offspring mice, nicotine readily crosses the placental barrier (60), and one can thus use maternal cotinine as an indicator of offspring nicotine exposure.

2.3 Behavioral Testing

After mice developed into adulthood (>9 weeks), I performed the following behavior assays in order : Open field arena (OF; anxiety, locomotion), elevated plus maze (EPM; anxiety), forced swim test (FST; depression), and sucrose anhedonia (depression). The first three tests occurred in increasing order of perceived stress. Although I viewed sucrose anhedonia as a comparatively stress-free assay, I performed this test last due to its multiple-day procedure. Open field and elevated plus maze tests were completed during the same week, but forced swim testing occurred one week later and sucrose anhedonia at least 1 week following that to ensure that any stressful effects of testing were resolved at that time.

OF, EPM, and FST occurred between zeitgeber time (ZT) 6 and ZT 10 to avoid any circadian related differences in behavior. Mice were given 30 minutes to acclimate to the testing

room prior to running the assays. Males and females were tested separately, and if both sexes were tested on the same day, the testing room was thoroughly cleaned and mice were introduced to the testing room at least an hour after the first sex left. Experimenters left the room during every trial.

2.3.1 Open Field Arena

Individual mice were placed into 4 identical 40 x 40 cm transparent acrylic chambers and were given 10 minutes to explore this novel environment. Because mice are a prey species that tend to avoid open spaces and have a strong explorative drive, this test can be used to analyze anxiety phenotypes. Two infrared beams formed a grid to detect motion in the 3D space, which was automatically analyzed with Photobeam Activity System (PAS) software (San Diego Instruments, San Diego, CA). Thus, I was able to measure a mouse's preference for the periphery or center, upward rearing movements, and total locomotion. Data was divided into 2 minute bins for analyses. More time spent in the periphery is indicative of an "anxious phenotype," whereas more time spent in the center is a "less anxious" phenotype. In between trials, every chamber was cleaned with 70% ethanol, soapy water, and then given fresh bedding. (Described in 1)

2.3.2 Elevated Plus Maze

Individual mice were placed onto the center a raised platform in the shape of a plus-sign and were given 5 minutes of exploration. Two of the arms were enclosed by walls, while the other two arms were open. The open arms pose a risk for falling, and thus mice instinctually gravitate in the closed arms. Therefore, more time spent in the open arms indicate a less anxious phenotype. The platform was cleaned with 70% ethanol and soapy water in between trials.

An overhead camcorder recorded every trial, and a blinded observer analyzed the videos for time to enter either arm after center placement, time spent in open arms, time spent in closed arms, and number of arm entries (Described in 1). The software used for analysis was Observer XT 8.0 (Noldus Information Technology, Leesburg, VA, USA).

2.3.3 Forced Swim Test

Two 3000 mL beakers were filled with ~2000 mL of water at 22-25 degrees celsius. An opaque barrier stood between the two beakers. Every trial an individual mouse was placed into one of the beakers, while a camcorder recorded their swimming for 6 minutes, but only the last 4 minutes were analyzed. A blinded observer scored the behavioral despair in terms of swimming/ climbing latency, floating latency, and time to first bout of floating. This test is used to observe an animal's response to a stressful environment, with floating behavior being a marker for learned-helplessness. Increased time floating indicates a depressive phenotype (69).

2.3.4 Sucrose Anhedonia

Mice were individually housed and given 1 day of acclimation prior to testing. Immediately before the active phase of ZT 14-20, water and food was removed from the cage and replaced with two 15 mL water bottles. One bottle contained 2% sucrose and the other contained filtered water. Water bottles were weighed before and after the testing period, cages were replaced with normal food and water, and then the entire procedure was repeated for a consecutive day. Water bottles were placed on the side opposite to the position on the first day to control for a side bias. Any cage replacements and water bottle weighings that occurred towards the end of the active phase were performed under dim red light. Males and females were tested separately. By measuring the water bottle weights, I could determine the amount of sweetened

water consumed compared to the amount of filtered water consumed. A weaker preference for the sweetened water indicated a “loss of pleasure” associated with depressive phenotypes (69).

2.4 Sleep

Following the analysis of OF testing, I found a significant difference within the males. Therefore, I decided to focus sleep analysis on male mice. Following Sucrose Anhedonia, I randomly selected 15 mice ($n_{pNic} = 8$, $n_{veh} = 7$) for sleep analysis.

2.4.1 Telemeter Implantation

As previously described by Borniger et al. (2015), F20-EET wireless transmitters (Data Sciences International (DSI), St. Paul, MN, USA) were implanted by an experienced surgeon using aseptic technique. Mice were deeply anesthetized and placed into a stereotaxic apparatus. The skull was exposed and cleaned, and two stainless steel screws (00-96 x 1/16; Plastics One, Roanoke VA, USA) that would serve as cortical electrodes were inserted through the skull to contact the dura mater. The screws were placed opposite and contralateral from one another, allowing the transmitter to record electric potential differences between the opposite regions of the two hemispheres. The transmitter was inserted into a subcutaneous pocket along the back of the animal, and another set of leads was inserted into the cervical trapezius muscles for EMG measurement. Animals were given post-operative analgesia and underwent 2 weeks recovery time. EEG/EMG data, temperature, and activity data were collected into a computer running Ponemah Software v6.3 (DSI). (Full details of surgery described in 3)

2.4.2 Sleep recording and analysis

Sleep was recorded, bandpass filtered (EEG: 0.3-25 Hz; EMG 25-50 Hz) and analyzed by 10-s epochs using NeuroScore Software v3.2 (DSI)**.** The delta band was set to 0.5-4 Hz and theta

was set to 6-9 Hz. All researchers avoided the experimentation room during sleep recordings to prevent any disturbances. Recording occurred during the 2 day baseline period, following sleep deprivation (*2.4.3*), and following LPS administration (*2.4.4*). Awake state was defined as high frequency EEG and high EMG. Rapid Eye Movement (REM) sleep was defined as high EEG with predominant theta frequencies (>2.5 theta/delta ratio) and low EMG. Non-REM (NREM) sleep was defined as low frequency, high voltage EEG and low EMG.

For spectral analyses, segments of baseline or recovery sleep following sleep deprivation were subjected to Fourier transformation, and periodogram data were cleaned of artifacts and organized by vigilance state. Spectral power in each vigilance state was plotted from 0.5-25 Hz. For data normalization, spectral power during baseline was subtracted from data generated following sleep deprivation at the same time of day.

2.4.3 Sleep deprivation

Sleep deprivation experiments can quantify the plasticity of an animal's circadian system by analyzing the rebound sleep. From ZT 0-5, I remained in the experimentation room and monitored both the mice and their live biopotential activities. Using gentle handling, mice were deprived of sleep during the entire 5 hour period of inactive phase, disturbing the cage whenever I noticed signs of sleep. Later analysis showed approximately 80% deprivation. Mice were allowed 18 hours of recovery, in which they received no disturbances.

2.4.4 LPS administration

Lipopolysaccharide (LPS) elicits an immune response in animals, which can include increased delta EEG power spectra in NREM sleep (39). To assess whether perinatal nicotine exposure alters an animal's sleep-related immune response, mice were injected (IP) with 0.5mg/

kg LPS in PBS thirty-two hours after sleep deprivation at ZT 13. EEG/EMG were recorded and analyzed during the following 24 hours.

2.5 Statistics.

Two-tailed t-tests were used to compare group means. Pre- and post-LPS spectral comparisons were completed using 2-way ANOVA with timepoint (pre- or post-LPS) and treatment (veh vs. pNic) as independent variables followed by Fisher's LSD post-hoc test. Statistical analyses were completed using SPSS Version 23 (IBM, Armonk, NY) or GraphPad Prism 6 (GraphPad Software, La Jolla, CA). A p value of ≤ 0.05 was considered statistically significant.

3. Results

To verify that mice exposed to nicotine perinatally received similar amounts of the drug, serum from the mothers was collected at the time of weaning and cotinine concentrations were determined. Maternal levels of cotinine were 0.2 ± 0.402 ng/ml for vehicle treated and 11.08 ± 0.364 ng/ml for nicotine-treated mice ($t = 47.16$, $p < 0.0001$) (**Fig. 1, B**). These cotinine values indicate that mice were receiving a stable level of nicotine, and cotinine concentrations were consistent with previous reports of light-smoking during pregnancy (57).

3.1 Behavioral phenotype in adulthood.

Male (but not female) mice that received nicotine perinatally had significantly reduced locomotor behavior in the OF test (**Fig. 2**). This was not associated with anxiety-like behavior, as central tendency in the open field was unchanged between groups ($p > 0.05$). Additionally, no

behavioral changes on the EPM were detected, indicating that perinatal nicotine exposure did not confer long-lasting changes in anxiety-like behavior in either male or female offspring (**Fig. 3**; $p > 0.05$). No changes were detected in sucrose preference (2%) on either testing day or in floating behavior in the FST, indicating that perinatal nicotine did not confer changes in depressive-like behavior into adulthood (**Fig. 3**; $p > 0.05$ in all comparisons).

3.2 Altered sleep in male mice exposed to perinatal nicotine.

After observing altered locomotor behavior in a novel open field environment, male mice were selected for sleep analysis. A randomly selected subset of male mice, equally distributed across treatment groups was chosen for these tests. After recovery from EEG/EMG telemeter implantation surgery (2 wk), the baseline phenotype was examined for 2 days. No changes were observed in locomotor activity or subcutaneous temperature values over time, and nicotine-exposed mice showed normal EEG traces (**Fig. 4A**; $p > 0.05$). Mice exposed to perinatal nicotine exhibited reduced wakefulness and increased NREM sleep during the inactive phase (**Fig. 4, D and E**). Despite increased time spent in NREM sleep, the delta (0.5–4 Hz) component of NREM sleep, an index of prior sleep pressure, showed normal patterns of activity over time, and whole day analyses of vigilance-state specific EEG spectra were unchanged between groups (**Fig. 5**; $p > 0.05$).

To further investigate the sleep plasticity, mice underwent a 5-h period of total sleep deprivation, and sleep time and NREM spectral components were analyzed during different portions of the recovery period (18 h and 6 h, respectively). Mice exposed to perinatal nicotine slept more during the recovery period and had a stronger increase (compared with their own baseline ZT-matched recording period) in NREM spectral power, specifically in the low delta

(0.5–1 Hz) range (**Fig. 6**; $p < 0.05$). This suggests that perinatal nicotine altered the homeostatic response to sleep loss in adulthood.

To assess whether these mice showed normal EEG responses to a bacterial immune challenge, they were injected (intraperitoneally) with 0.5 m/kg LPS 1 h before the start of the active (dark) phase (~ZT 13), and EEG/EMG biopotentials were monitored for the following 24 h. Mice that did not receive perinatal nicotine increased EEG delta frequencies during the hours following LPS administration, while those exposed to perinatal nicotine had a largely blunted response (**Fig. 7**; $p < 0.05$).

4. Discussion

Our results show that perinatal nicotine exposure indeed alters behavior in offspring in adulthood. Although no anxious or depressive phenotype was present, pNic male mice had reduced locomotion during Open Field Exploration. However, EEG/EMG analysis revealed no difference in total locomotion between the two treatment groups across the circadian cycle, notably during the testing period of ZT 6-10 (**Fig. 4, B**). The strongest significant difference in total movement occurred during the first two minutes of Open Field Exploration (**Fig. 2, A**). Therefore, some aspect of the initial novel environment presentation induced this change in movement. Additional behavioral testing could elucidate a better explanation for this behavioral phenotype. For example, repeated exposures to the Open Field apparatus could reveal differences in habituation abilities between pNic and veh males (1). Other tests of novelty-seeking could have also been employed, such as Novel Object Exploration, Light/Dark Chamber Test (both described in 55), or Social Novelty (described in 79). Although nAchR's are expressed at the

level of neuromuscular junctions (35), it is unlikely that altered muscle activity contributed to the locomotor phenotype, as nicotine has low affinity for nAChRs at the neuromuscular junction (78), as well as the lack of significant differences during the physically strenuous Forced Swim Test in this experiment (**Fig. 3, C and F**).

More notably, this study demonstrates long-lasting changes in sleep following chronic perinatal nicotine exposure. Perinatally-exposed males spent more time in NREM sleep during the inactive phase, less time awake during the active phase, and had an exaggerated homeostatic response to sleep deprivation.

Several mechanisms may underlie these changes in sleep. Perinatal nicotine may affect the development of biological rhythms, as evidenced by its activation of fetal clock regions (7). The mother's suprachiasmatic nucleus (SCN) mediates the synchronization of maternal-fetal biological rhythms (10, 21, 11) while the fetal retinohypothalamic tract (RHT) is still developing (61), so any alterations in cholinergic signaling of the maternal clock may lead to downstream changes in the fetal clock. The cholinergic system's involvement in biological rhythms may be a mechanistic target. Both the RHT and SCN — two major components of circadian entrainment — express nAChR (52). Fetal nicotine exposure alters nAChR expression in the neonatal hypothalamus (36), which contains the SCN. In fact, nicotine's effects on the hypothalamus may be a major contributor to the altered phenotype observed in sleep homeostasis (76). Moreover, light entrainment increases nicotinic signaling in the SCN (45).

Dopamine signaling, as well, is important for synchronization of maternal-fetal biological rhythms (74). Dopaminergic midbrain regions express nAChRs (6), and thus the altered dopaminergic signaling resulting from nicotine administration (44) may over-activate this

maternal-fetal synchronization signal. Indeed, the ventral tegmental area (VTA), contributes to the maintenance of sleep-wake states (12, 13), with orexin/hypocretin fibers innervating cells of this heavily dopaminergic region (49). Individuals who are prescribed drugs that alter dopaminergic signaling — such as those with Parkinson's Disease, ADHD, and schizophrenia — may have altered sleep behaviors following treatment (29, 9, 66, 46). Sleep deprivation, as well, predisposes individuals to increased reward circuitry activity and behavior (47). In conclusion, there are several demonstrations that nicotine's primary neurotransmitter activity — being acetylcholine and dopamine — contribute to biological rhythms in sleep. It is possible that these systems act separately to alter sleep, or they may act in tandem.

Alternatively, perinatal nicotine exposure may disturb autonomic physiology to alter sleep. Nicotinic receptors are indeed expressed on brainstem regions that regulate the autonomic nervous system (77, 65). Gestational nicotine aggravates chemoreception and breathing patterns (14, 8), which could contribute to hypersomnia. This nicotine exposure also affects heart-rate and autonomic control (18). It is possible that nicotine directly targets the maternal autonomic nervous, which leads to subsequent alterations in that of the fetuses, or nicotine could target the fetal autonomic nervous system as well.

Additional manipulation of cholinergic and/or dopaminergic signaling in vigilance regions can help determine a more specific mechanism by which nicotine affects offspring sleep. Orexin/hypocretin is produced in the lateral hypthalamus, and is classically linked to behaviors regarding circadian rhythmicity, feeding, and wakefulness (82). Orexin signaling also contributes to nicotine's rewarding properties (27). Orexin cells express many subtypes of nAChRs, including $\alpha 4\beta 2$ (40). Knock-out mice for these receptor subunits show reduced arousal to

nicotine and subsequent homeostatic rebound, and antagonizing α4 subunits in the orexigenic regions of the hypothalamus increased feeding following a fast compared to controls (40, 56, 22). α7-Containing subunits, as well, may provide insight, as these receptors are upregulated in arousal/homeostatic brain regions (basal forebrain and hypothalamus) in early post-natal life following perinatal nicotine exposure (20), and also contribute to dopaminergic signaling in the VTA (62). Furthermore, both α4β2 and α7 expressed on the medulla in the brainstem modulate nicotine's effects on autonomic physiology (16). Taken together, the SCN, lateral hypothalamus, VTA, and brainstem are regions worth exploring, with a focus on α4β2 and α7 nAChR subunits. Future experiments could include combining perinatal nicotine treatment with antagonists of various nAChR subtypes, or with conditional/tissue-specific knock-out animals that do not express these subtypes.

To my knowledge, this is the first study to explore the immune-induced sleep response as a result of perinatal nicotine exposure. pNic males exhibited a weaker response to LPS administration, characterized by the lack of increase in deep sleep. It is possible that *in utero* exposure to nicotine created long-lasting suppressions of the offspring's immune systems. Indeed, perinatal nicotine exposure reduces immune cell responsiveness and proliferation in offspring (2, 50). Mice exposed to E-Cig nicotine vapor had an attenuated immune response to bacterial and viral infections, which was associated with defective macrophage clearance and higher levels of IL-6 and MCP-1 (70). Nicotine also hinders the ability of dendritic cells to respond to pathogens and stimulate T-cells (51), while additionally up-regulating neutrophil extracellular traps (30), which in excess could induce an autoimmune reaction (37). Indeed, α7 nAChR subunits are expressed on immune cells (33, 63) and its stimulation lowers TNF-α and

IL-1β release (75, 58). All considered, nicotine likely directly altered cholinergic signaling within the offspring immune system. To further explore this hypothesis, one could examine other immune responses between pNic and veh mice. For example, LPS administration also induces an increase in depressive-like behaviors (53) and hypothalamic-pituitary-adrenal axis production of stress hormones (25). Examining these immune responses could show whether perinatal nicotine attenuates other sickness behaviors. Further analysis of spleen (41) and adrenal weights (25) could reveal the extent to which perinatal nicotine exposure alters immune organs.

5. Significance

In conclusion, males exposed to perinatal nicotine displayed reduced locomotion after placement into a novel environment, which may indicate a reduced-exploratory phenotype. They also spent more time asleep and less time awake, required a stronger and longer NREM rebound following sleep deprivation, and had a reduced somnogenic response to immune challenge. Because I administered pure nicotine salt through drinking water, these effects can be attributed to nicotine and not other compounds present in smoking tobacco, e-cigarette juice, etc. Thus, the results can generalize to all forms of nicotine. I ended nicotine administration immediately after weaning, so the effects I observed in adulthood are attributed to long-lasting changes from developmental nicotine exposure. Overall, I believe that perinatal nicotine exposure is altering nAChR and dopamine receptor expression in brain regions associated with autonomic and vigilance signaling, or transient changes in early life are leading to more long-term downstream changes elsewhere. In addition, perinatal nicotine exposure likely targets nicotinic receptors on fetal immune cells, which leads to downstream changes in cytokine production and proliferative

ability of these cells. This reduced peripheral immune response would then have more difficulty signaling the central nervous to induce sickness behaviors, including hypersomnia.

With the ongoing prevalence of smoking during pregnancy and risk of second-hand smoke, this study establishes the risk for dysfunctional sleep in offspring. It also shows that e-cigarettes, nicotine patch, and other forms of replacement-therapy are not safer alternatives during pregnancy. Additionally, many neurotoxic organophosphates alter cholinergic activity (64), so results may even generalize to certain environmental toxins.

Future studies should elucidate a mechanism, as well as whether these effects are seen in females as well. Additionally, it is important to track pNic-induced sleep changes throughout the lifetime. These discoveries could lead to treatment for the sleep issues resulting from perinatal nicotine exposure, and the resulting health problems that may be associated with altered sleep.

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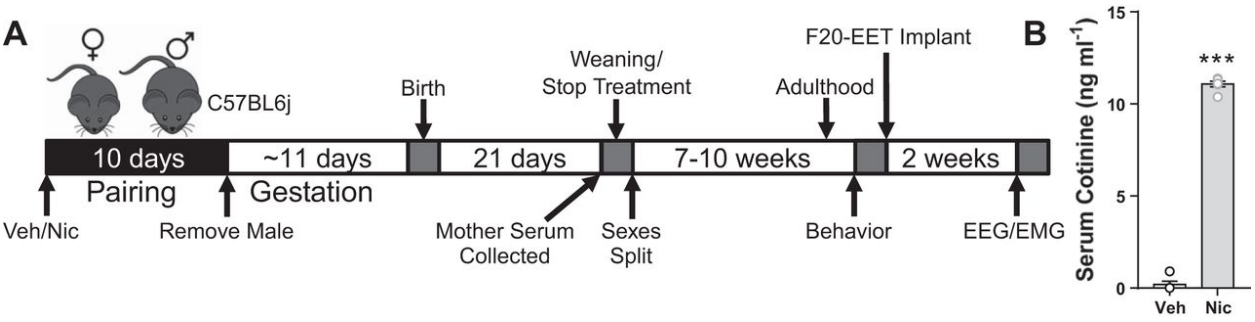

Figure 1

Experimental design. A: adult female and male C57BL6j mice were paired for 10 days to allow multiple mating opportunities. At this time, water bottles were replaced with ones containing 200 µg/ml nicotine bitartrate dihydrate in 2% wt/vol saccharin (nic) or pH-matched 2% saccharin with 0.2% tartaric acid (vol/vol) (veh). Treatment continued until weaning (total 6-wk exposure). The stud male was removed 10 days postpairing. At weaning (postnatal day 21), mother serum was collected to assess circulating cotinine concentrations, and male and female offspring were separated and singly housed. Treatment was stopped at this time and mice were given ad libitum access to normal drinking water. Seven to ten weeks later, mice underwent behavioral testing, and then a randomly chosen subset of males was chosen for EEG/ EMG sleep analyses. B: nicotine-exposed mothers had higher cotinine levels in their serum at weaning, indicating that the mice were indeed being exposed to significant levels of nicotine ($t_9 = 47.16$, ***$P < 0.0001$, Student's two-tailed t-test; n = 5 veh, 6 Nic mothers, error bars represent SE).

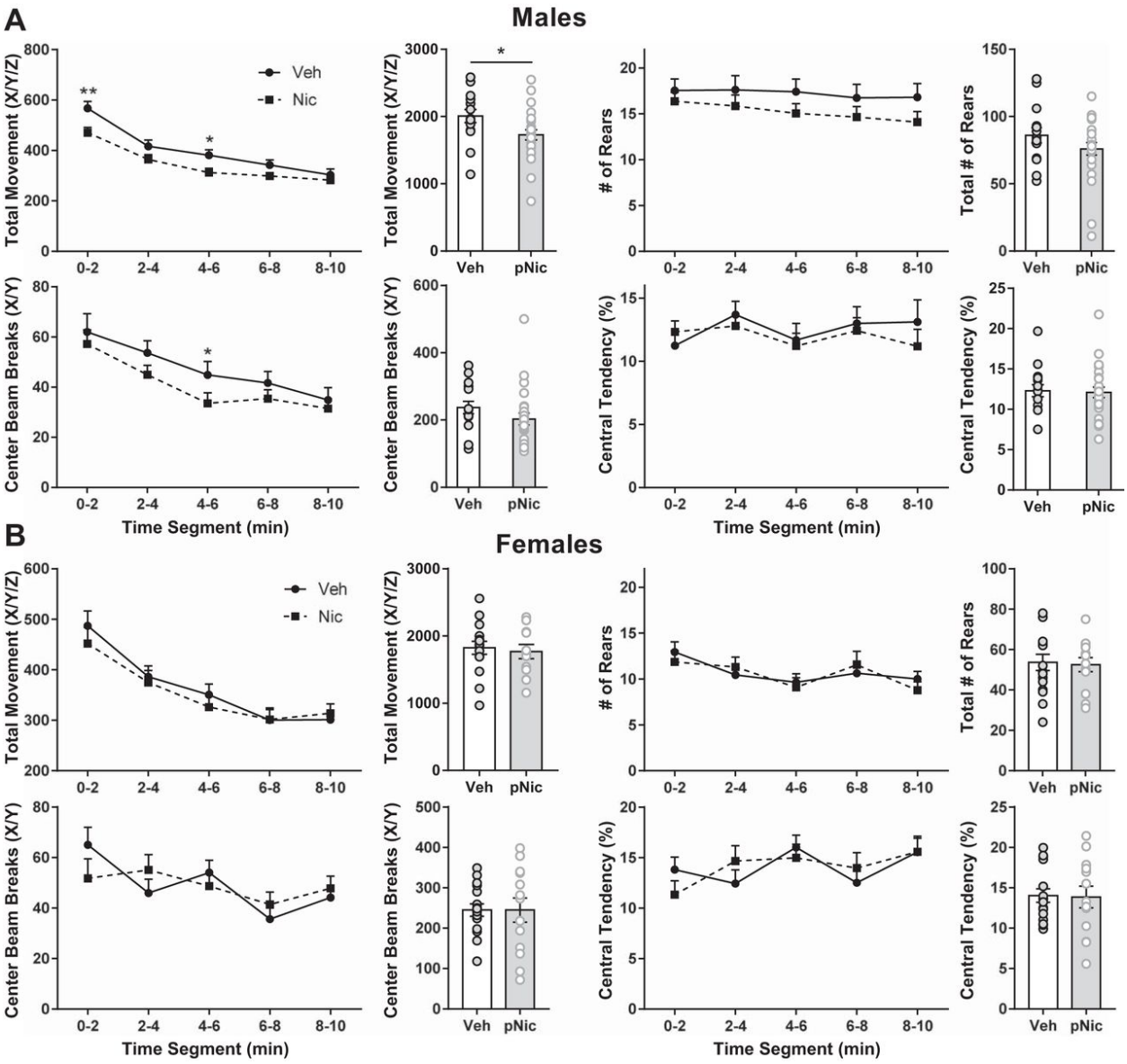

Figure 2

Males exposed to perinatal nicotine have reduced locomotion in a novel open field environment. A: males showed reduced total movement over the course of a 10-min open field test (10 min total: $t38 = 2.828$, $P = 0.0282$); however, they did not show anxiety-like behavior as central tendency was unchanged between treatment groups. B: this locomotor deficit in a novel environment was not evident in female mice, which had no discernible change in the open field between treatment groups (males: n = 15 veh, 25 pNic, females: n = 16 veh, 13 pNic, error bars represent SE, $^{**}P < 0.01$, $^{*}P < 0.05$ Student's two-tailed t-test).

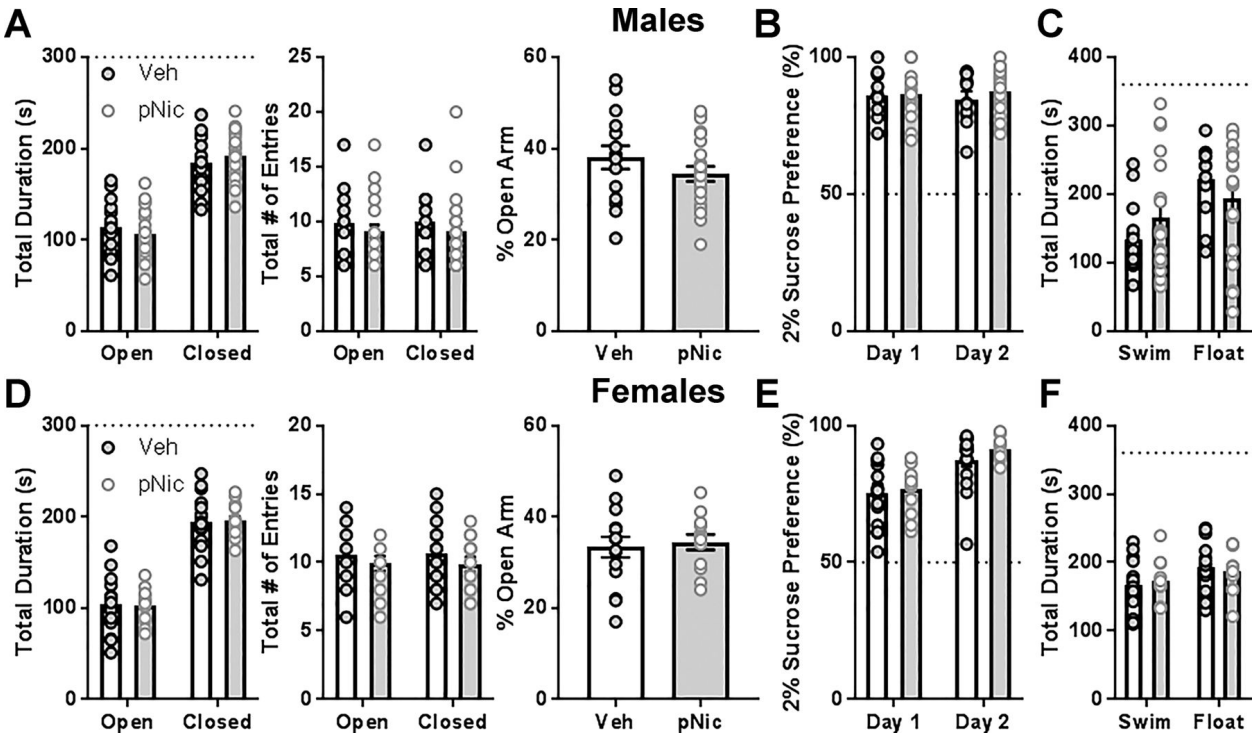

Figure 3

Perinatal nicotine exposure does not alter behavior on the elevated plus maze, sucrose preference, or forced-swim tests in adulthood. A and D: total time spent in the open and closed arms of the elevated plus maze in the 5-min test (max denoted by dotted line), total number of entries into either open or closed arm, and percent time spent on the open arm. B and E: sucrose preferences on 2 consecutive days of testing. C and F: total swimming and float durations in the 6-min forced swim test (dotted line denotes maximum) (males, n = 15 veh, 23 pNic; females, n = 13 veh, 16 pNic; error bars represent SE).

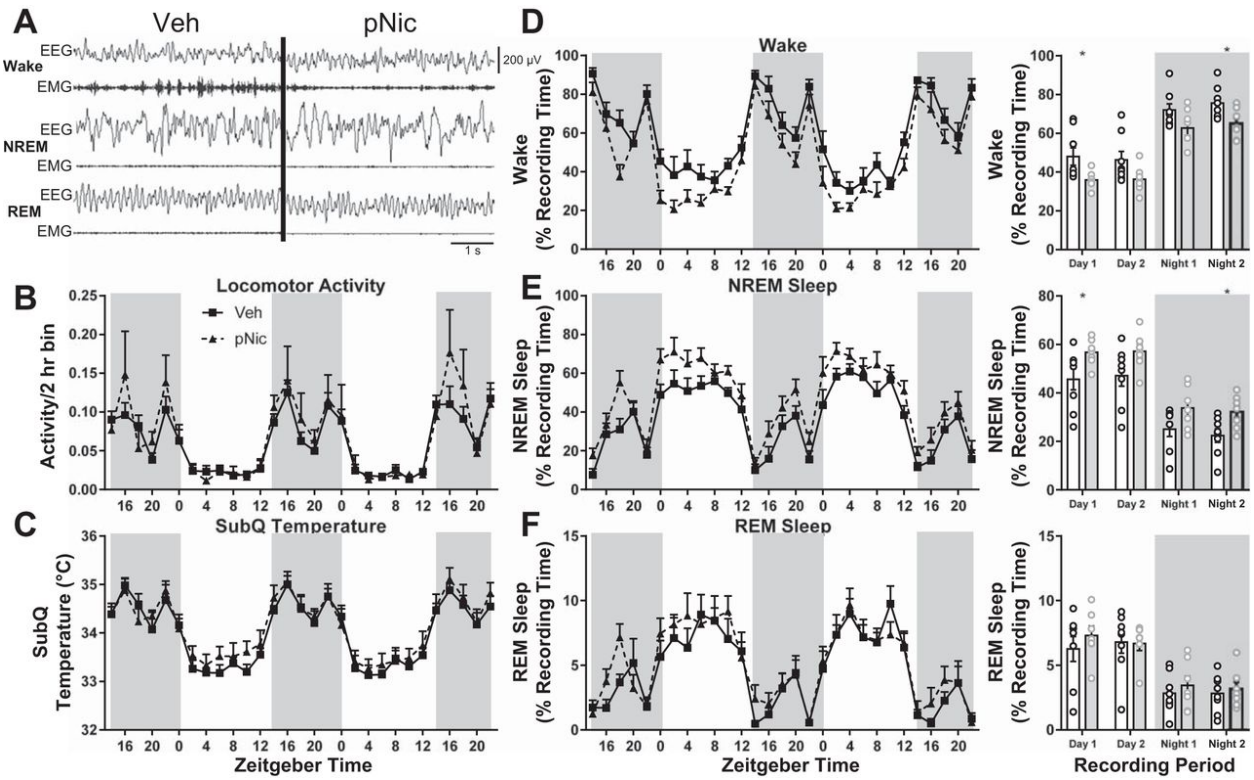

Figure 4

Perinatal nicotine exposure has long-lasting effects on sleep-wake states into adulthood in male mice. A: representative EEG/EMG traces from vehicle (left) and (right) perinatal nicotine-exposed (right) mice. No differences were detected in spontaneous locomotor activity (B) or subcutaneous temperature rhythms (C). However, changes in the amount of time spent (D) awake (two-way ANOVA main effect of time: $F_{3,39} = 69$, $P < 0.0001$; main effect of treatment: $F_{1,13} = 8.349$, $P = 0.0127$; day 1 $t_{13} = 2.406$, $P = 0.032$; night 2 $t_{13} = 2.463$, $P = 0.029$), in non-rapid eye movement (NREM) sleep (two-way ANOVA main effect of time: $F_{3,39} = 68.83$, $P < 0.0001$; main effect of treatment: $F_{1,13} = 6.849$, $P = 0.0213$; day 1 $t_{13} = 2.219$, $P = 0.045$; night 2 $t_{13} = 2.518$, $P = 0.026$) but not in REM sleep were observed (n = 7–8/group, error bars represent SE, *$P < 0.05$ by Student's two-tailed t-test).

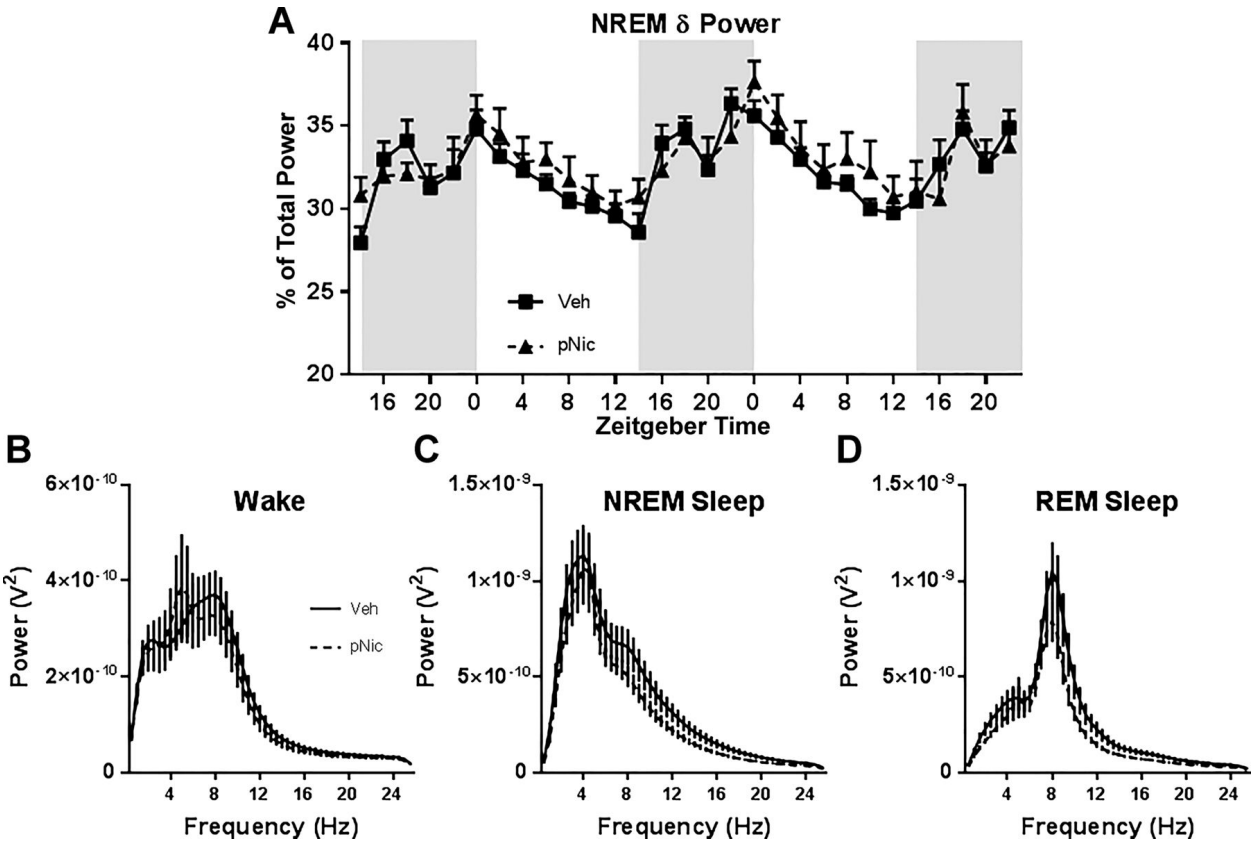

Figure 5

Perinatal nicotine does not alter NREM sleep microstructure or the EEG spectra within wakefulness, NREM or REM sleep. A: NREM delta power over time as a percent total EEG power within each 2-h bin. Power spectra of the 2 baseline days for wakefulness (B), NREM sleep (C), and REM sleep (D) were not different between groups (n = 8 veh, 7 pNic; error bars represent SE).

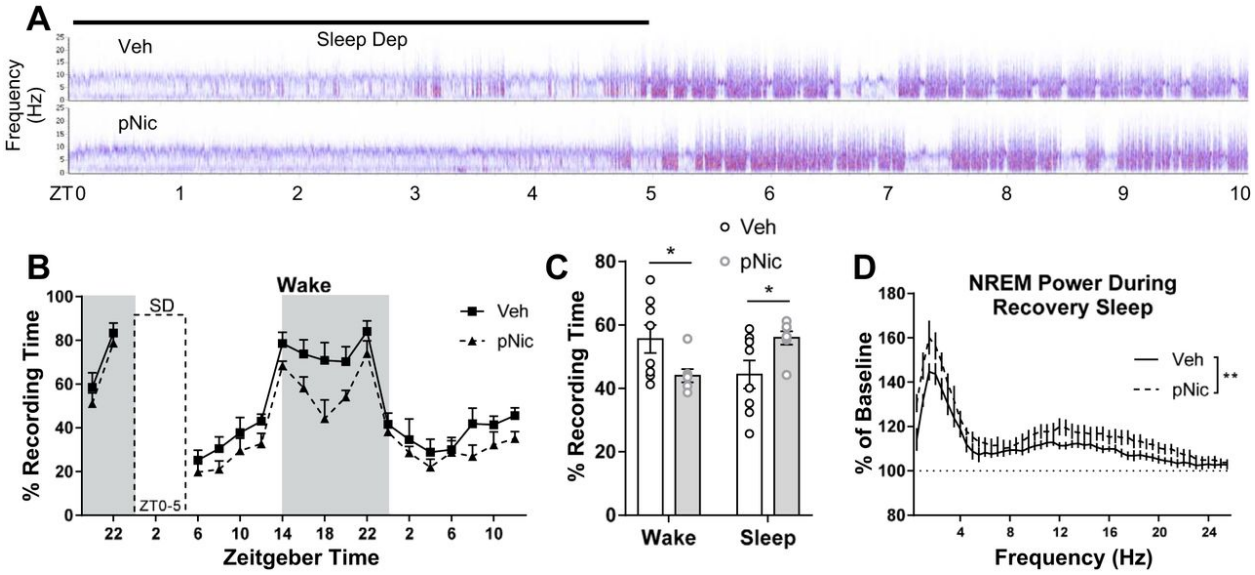

Figure 6

Perinatal nicotine exposure alters the homeostatic response to sleep deprivation. A: representative EEG spectrograms from a vehicle and nicotine-treated mouse during and following 5-h sleep deprivation. Total wake time following sleep deprivation (B) and (C) total wake and sleep times during the 18-h recovery period (C) (wake $t_{13} = 2.251$, $P = 0.042$; sleep $t_{13} = 2.248$, $P = 0.0426$) normalized (to NREM power during the same time period of baseline recording) NREM spectral power during the first 6 h of recovery sleep (D) [zeitgeber time (ZT) 5–11; whole spectrum average $t_{100} = 2.635$, $P = 0.0097$; n = 8 veh, 7 pNic, error bars represent SE, *$P < 0.05$, **$P < 0.01$ by Student's two-tailed t-test].

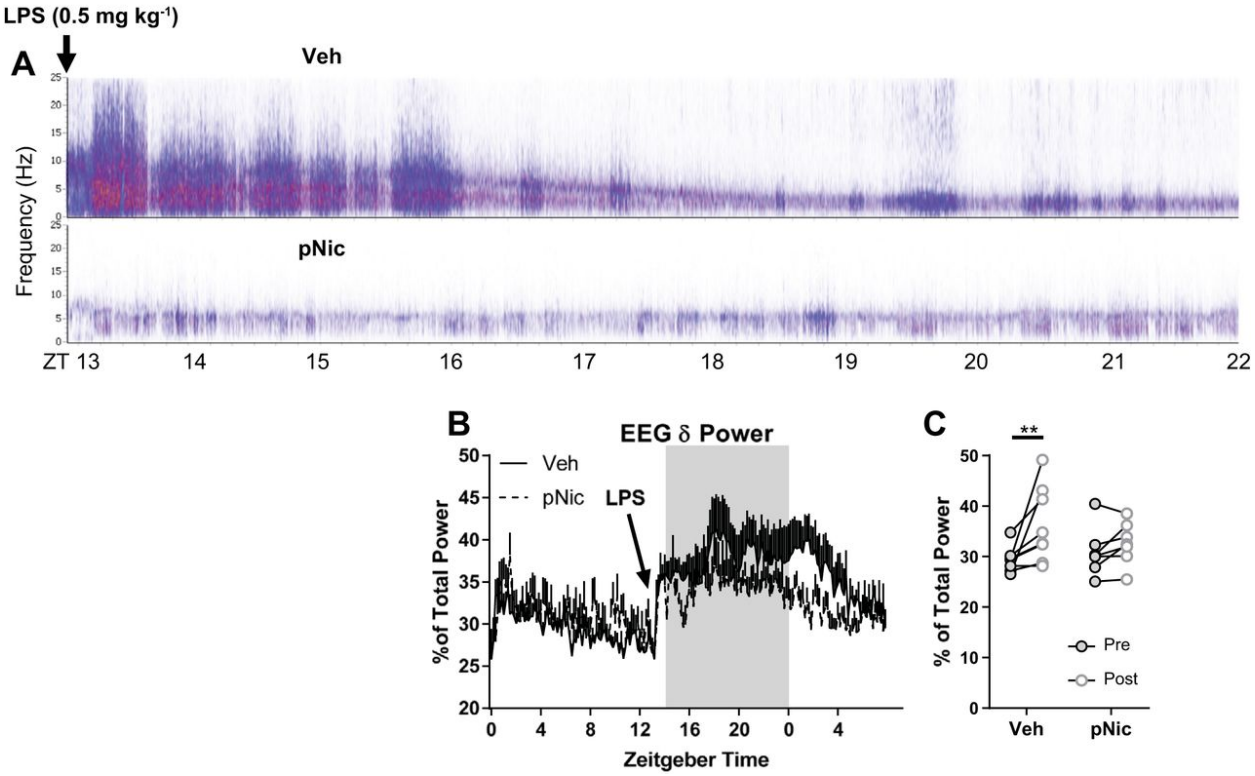

Figure 7

Perinatal nicotine alters the EEG slow-wave response to intraperitoneal LPS. A: representative EEG spectrograms from a veh (top) and pNic (bottom) treated mouse during 10 h following intraperitoneal LPS (0.5 mg/kg) injection (denoted with arrow). In the veh-treated mouse, an obvious frequency decline into the delta (0.5–4 Hz) range is evident following LPS, where the same pattern is absent in the mouse give pNic. B: summed (10 min binned) data over time showing the delta band over time as a percent of total spectral power before and following LPS. C: pNic prevented LPS-induced increases in EEG delta power, as shown by trajectory plots pre- and post-LPS injection. A significant difference was detected in the veh mice but not in pNic-treated animals (two-way ANOVA main effect: of pre- vs. post-LPS $F_{1,13} = 8.798$, $P = 0.0109$; Sidak's multiple comparisons post hoc test: veh-pre vs. veh-post-LPS $t = 3.446$, adjusted $P = 0.0087$; n = 8 veh, 7 pNic, error bars represent SE, $^{**}P < 0.01$).

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